

# Co-localization and functional coupling of creatine kinase B and gastric H<sup>+</sup>/K<sup>+</sup>-ATPase on the apical membrane and the tubulovesicular system of parietal cells

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Immunogold labelling of creatine kinase B (BB-CK) and gastric H<sup>+</sup>/K<sup>+</sup>-ATPase in the parietal cells of the stomach revealed co-localization of these two enzymes on the apical membrane and the membranes of the tubulovesicular system. Upon fractionation of hog parietal cells, a specific fraction of the BB-CK proteins remained associated with the purified vesicles, in which gastric H<sup>+</sup>/K<sup>+</sup>-ATPase is highly enriched. The BB-CK present in this highly purified preparation was able to support pronounced H<sup>+</sup>/K<sup>+</sup>-ATPase activity in K<sup>+</sup>-loaded vesicles in the presence of phosphocreatine and ADP, although only low levels of ATP

were measured. In contrast, when pyruvate kinase, phosphoenolpyruvate and ADP were used as an ATP-generating system to sustain similar levels of H<sup>+</sup>/K<sup>+</sup>-ATPase activity, ATP levels were more than 10-fold higher. Changing the experimental conditions such that ATP levels were the same for both systems resulted in significantly elevated H<sup>+</sup>/K<sup>+</sup>-ATPase activities in the BB-CK/phosphocreatine system in comparison with the pyruvate kinase/phosphoenolpyruvate system. These results indicate that gastric H<sup>+</sup>/K<sup>+</sup>-ATPase has preferential access to ATP generated by creatine kinase co-localized on the membranes of the vesicles.

## INTRODUCTION

The creatine kinase/phosphocreatine (CK/PCr) system consists of a small family of isoenzymes which catalyse the reversible exchange of high-energy phosphate groups between PCr and ADP through the reaction:  $\text{MgADP}^- + \text{PCr}^{2-} + \text{H}^+ \rightleftharpoons \text{MgATP}^{2-} + \text{creatine}$ . Four CK (EC 2.7.3.2) subunits are known to exist in mammals [1]. Two of them [B-CK (brain) and M-CK (muscle)] are restricted to the cytosol. These isoforms are enzymically active as BB-CK or MM-CK homodimers, or as MB-CK heterodimers [1]. The other two subunits, ubiquitous Mi<sub>g</sub>-CK and sarcomeric Mi<sub>s</sub>-CK, are restricted to the mitochondria [2]. An important function of the system is to maintain temporal and spatial energy buffers in cells that consume large amounts of ATP. This 'CP-shuttle' [3] was first proposed to transfer high-energy phosphoryl groups from sites of production to sites of energy consumption in skeletal muscle [4], but is now known to play a role in other polarized cell types, such as photoreceptor cells of the retina [5,6] and mature spermatozoa [7,8]. Furthermore, the CK/PCr system is thought to assist in the maintenance of ATP/ADP ratios at sites of ATP hydrolysis, thereby avoiding the inactivation of ATPases and the loss of adenine nucleotides [9]. The observation that the CK/PCr system is functionally coupled to several types of ATPases supports this concept. This coupling has been clearly demonstrated for Na<sup>+</sup>/K<sup>+</sup>-ATPase [10,11] and sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase [12,13], two members of the class of P-type ATPases which is characterized by phosphorylation of the catalytic subunit by ATP [14].

Here we report on another member of the P-type ATPases, gastric H<sup>+</sup>/K<sup>+</sup>-ATPase (EC 3.6.1.36), which is the main protein involved in the high-energy-demanding process of gastric acid secretion [15]. This enzyme consists of a catalytic 114 kDa  $\alpha$ -subunit and an extensively glycosylated  $\beta$ -subunit. It is located in the apical membrane and in the membranes of the tubulovesicular system of parietal cells [16]. H<sup>+</sup>/K<sup>+</sup>-ATPase transports protons into the lumen of the stomach in exchange for K<sup>+</sup> ions, thereby creating a pH gradient of up to 6 units. This gradient, in combination with K<sup>+</sup> and Cl<sup>-</sup> specific conductances, in turn leads to gastric hydrochloric acid secretion [17].

The high energy requirements of gastric acid secretion are reflected in the enormous numbers of mitochondria present in the parietal cells. In total, they occupy about 35% of the cytoplasm, which is more than in any other cell type [18]. Although it is conceivable that ATP is directly transported from the mitochondria to the gastric H<sup>+</sup>/K<sup>+</sup>-ATPase, we have assumed that there would be a supportive role for the CK/PCr system because of the similarity with other P-type ATPases and because there is a clear increase in the apparent equilibrium constant ( $K' = [\text{PCr}][\text{ADP}]/[\text{Cr}][\text{ATP}]$ ) of the CK reaction during gastric acid secretion [19]. Furthermore, human, rabbit and mouse parietal cells have been shown to contain high levels of BB-CK [20,21]. Here we provide evidence from both morphological and cell fractionation studies that a membrane-associated subfraction of BB-CK co-purifies with gastric H<sup>+</sup>/K<sup>+</sup>-ATPase and that these two enzymes are located in close proximity in parietal cells. Furthermore, biochemical evidence is provided to demonstrate that gastric H<sup>+</sup>/K<sup>+</sup>-ATPase has preferential access to ATP

Abbreviations used: CK, creatine kinase; PCr, phosphocreatine; PK, pyruvate kinase; PEP, phosphoenolpyruvate; MoAb, monoclonal antibody; PoAb, polyclonal antibody; SCH 28080, (3-(cyanomethyl)-2-methyl-8(phenylmethoxy)imidazo[1,2-a]pyridine); PB, 0.1 M sodium phosphate buffer, pH 7.3; G6PD, glucose-6-phosphate dehydrogenase; AO, Acridine Orange; IEM, immuno-electron microscopy.

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generated by this subpopulation of BB-CK. Our results strongly support the hypothesis that there is a functional coupling between these two enzymes in the parietal cells of the stomach.

## MATERIALS AND METHODS

### Immuno-electron microscopy (IEM)

After dissection, small fragments of rabbit stomach were briefly rinsed in physiological saline and immediately fixed for 2 h at 4 °C in 0.1 M sodium phosphate buffer, pH 7.3 (PB) containing 2% paraformaldehyde and 0.1% glutaraldehyde. Tissue samples were stored in 1% paraformaldehyde in the same buffer. For IEM, small pieces of tissue were immersed in 2.3 M sucrose and immediately frozen in liquid nitrogen. Ultrathin sections were cut using glass knives at -100 °C with a Reichert Ultracut S. They were picked up on formvar-coated copper grids and transferred on to molten gelatin. Sections were rinsed three times in PB containing 0.1% BSA/0.1% gelatin/0.15% glycerine before incubation with anti-CK monoclonal antibody (MoAb) 20H3B (diluted 1:20000 in PB), developed as described in [22], or anti-H<sup>+</sup>/K<sup>+</sup>-ATPase MoAb 5B6 (diluted 1:5000 in PB) [23], either for 60 min at room temperature or overnight at 4 °C. After rinsing three times in PB, sections were incubated with rabbit anti-(mouse IgG) (25 µg of IgG/ml) for 45–60 min at room temperature. After extensive rinsing, sections were incubated in a Protein A-gold (10 nm particles) solution for 60 min [24] and then stained with 0.3% uranyl acetate in a 2% methylcellulose solution. Grids were dried following the removal of excess methylcellulose. Sections were examined with a Philips EM 301 electron microscope and photographed on 35 mm Kodak film.

For double labelling experiments, sections were treated as described above, with the following modifications. The primary incubation was performed with a mixture of anti-CK MoAb 20H3B (1:20000) and anti-H<sup>+</sup>/K<sup>+</sup>-ATPase polyclonal antibody (PoAb) HKB (1:500) [25]. After extensive rinsing, sections were incubated with a mixture of goat anti-(mouse IgG) coupled to 5 nm gold particles and goat anti-(rabbit IgG) coupled to 10 nm gold particles (Aurion, Wageningen, The Netherlands).

### Protein analysis

Ion-tight inside-out H<sup>+</sup>/K<sup>+</sup>-ATPase vesicles (1.5–2.0 mg/ml) were isolated as described previously [26]. The protein content of each fraction was determined according to a modified Lowry method [27]. Proteins were separated in 10% (w/v) polyacrylamide/bisacrylamide gels according to Laemmli [28] and electroblotted on to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) in 10 mM CAPS (3-cyclohexylamino-1-propanesulphonic acid) and 10% (v/v) methanol, pH 11.0, at 4 °C for 1 h at 100 V. Blots were preincubated for 2 h in PBS containing 1% (w/v) gelatin, before incubation with primary antibody (CK-specific MoAb 20H3B or H<sup>+</sup>/K<sup>+</sup>-ATPase-specific PoAb HKB) overnight in PBS containing 0.05% (v/v) Tween-20 and 1% (w/v) gelatin. Bound antibodies were detected using anti-species antibodies, followed by incubation with either peroxidase-conjugated mouse or rabbit anti-peroxidase antibodies (DAKO, Glostrup, Denmark), for MoAbs or PoAbs respectively. Bound conjugates were detected by incubation in PBS containing 10 mg/ml 4-chloro-1-naphthol, 17% (v/v) methanol and 0.015% (v/v) H<sub>2</sub>O<sub>2</sub>.

### CK activity measurements

The activity of CK was measured at 25 °C using the CK NAC-activated MPR I kit (Boehringer Mannheim), and calculated according to the specifications provided by the manufacturer.

### K<sup>+</sup>-efflux measurements

Purified H<sup>+</sup>/K<sup>+</sup>-ATPase vesicles were loaded with K<sup>+</sup> by incubation for 1.5 h at 37 °C in 5 mM Pipes/Tris, pH 7.0, 2 mM MgCl<sub>2</sub> and 125 mM KCl. Either 50 or 100 µl of K<sup>+</sup>-loaded vesicles was diluted in 2 ml of efflux medium [5 mM Pipes/Tris, pH 7.0, 2 mM MgCl<sub>2</sub>, 150 mM choline chloride and 10 µM Acridine Orange (AO)]. Care was taken to maintain ionic balance in the cuvette, through the addition of counterions. AO fluorescence was measured using a Shimadzu RF-510 spectrofluorophotometer in a stirred 3 ml cuvette at 20 °C, at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

### ATP assays

The rate of ATP synthesis was measured using the same reaction mixture as for the K<sup>+</sup>-efflux measurements, except for the addition of 5 mM glucose, 1 mM NADP<sup>+</sup>, 1.4 units/ml hexokinase and 0.7 units/ml glucose-6-phosphate dehydrogenase (G6PD). The rate of ATP synthesis was measured as the rate of NADPH synthesis detected at 340 nm on a PU 8620 spectrophotometer. ATP concentrations during the efflux reactions were measured in 200 µl samples from the reaction mixture; proteins were denatured by the addition of 100 µl of 3 M perchloric acid and precipitated by centrifugation at 12000 g for 5 min. The supernatant was neutralized by the addition of 100 µl of 3 M KOH and, after a second round of centrifugation, its ATP concentration was determined by chemiluminescence analysis on a LKB 1250 luminometer using firefly lantern extract (Sigma, Bornem, Belgium). ATP concentrations were determined from a standard curve prepared in the same way.

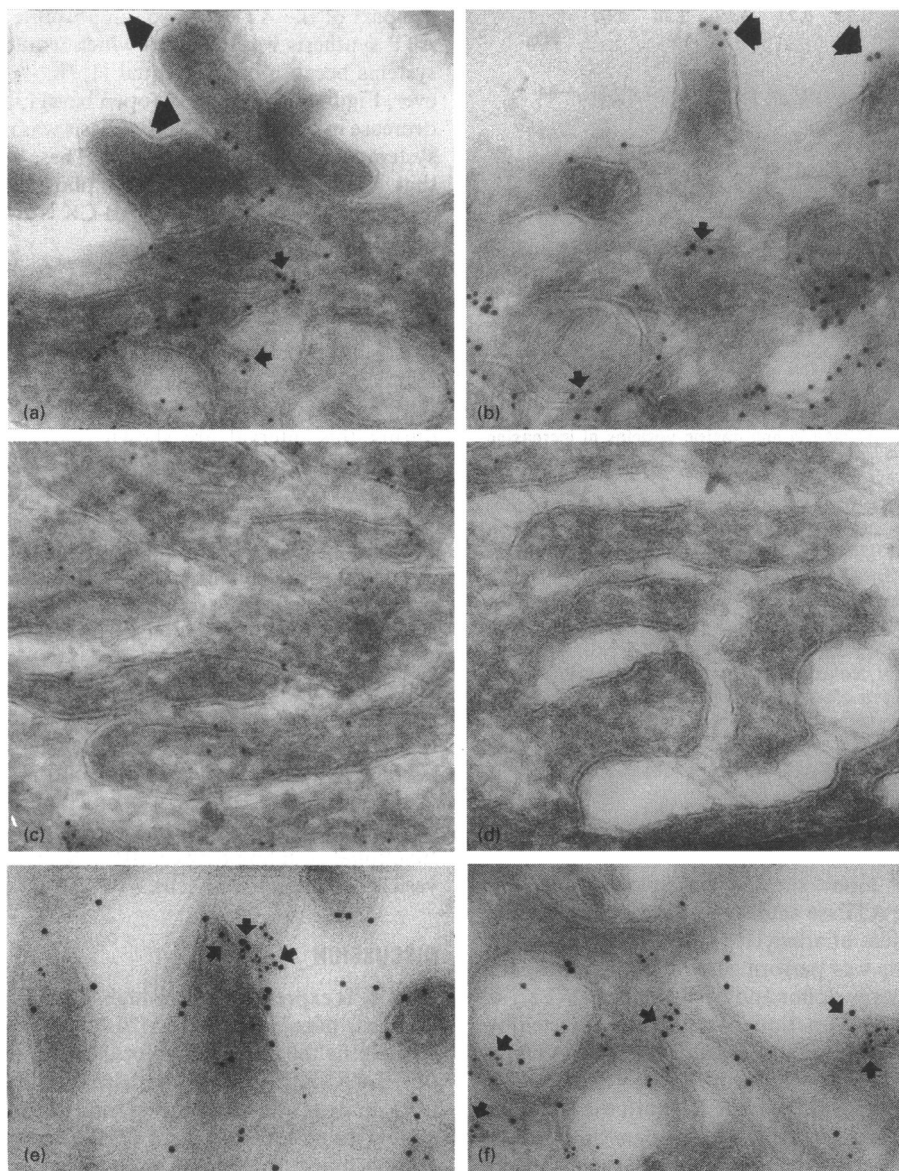
## RESULTS

### Co-localization of BB-CK and gastric H<sup>+</sup>/K<sup>+</sup>-ATPase in the parietal cells of the stomach

Electron microscopy with immunogold labelling was performed to obtain information on the intracellular distribution of BB-CK in the parietal cells of the rabbit stomach. Data as shown in Figures 1(a) and 1(c) reflect the ambiguous nature of BB-CK. IEM analysis of sections incubated with anti-CK MoAb 20H3B revealed the presence of unbound enzyme evenly distributed throughout the cytosol, in addition to BB-CK bound to both the apical membrane and the membranes of the tubulovesicular system, as well as to the basolateral membrane. Incubation of serial sections with MoAb 5B6, specific for the  $\alpha$ -subunit of H<sup>+</sup>/K<sup>+</sup>-ATPase [23], revealed this protein to be located exclusively on the apical membrane and the membranes of the tubulovesicular system (Figures 1b and 1d). Finally, double labelling experiments were performed using the CK-specific MoAb 20H3B in combination with PoAb HKB, recognizing the H<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit. The results reveal that at least 25% of the H<sup>+</sup>/K<sup>+</sup>-ATPase gold particles (10 nm gold) co-localize with the fraction of BB-CK (5 nm gold particles) bound to the apical membrane (Figure 1e) and to the membranes of the tubulovesicular system (Figure 1f).

### BB-CK in isolated vesicles of hog parietal cells

In order to obtain independent support for the association of BB-CK with the tubulovesicular system, hog gastric vesicles were isolated according to a cell fractionation protocol developed for the purification of gastric H<sup>+</sup>/K<sup>+</sup>-ATPase [26]. Western blotting of each of the successive fractions in the purification process with CK-specific MoAb 20H3B revealed the presence of BB-CK in all



**Figure 1** Intracellular distribution of BB-CK and  $H^+/K^+$ -ATPase in gastric parietal cells, as revealed by immunogold labelling

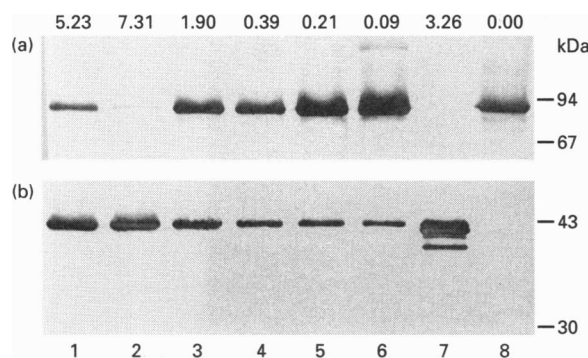
Sections were incubated with MoAb 20H3B or 5B6, specific for CK (**a, c**) and  $H^+/K^+$ -ATPase (**b, d**) respectively, or with a mixture of CK-specific MoAb 20H3B and  $H^+/K^+$ -ATPase-specific PoAb HKB (**e, f**). The distribution of BB-CK (**a**) and  $H^+/K^+$ -ATPase (**b**) is shown at the apical side of the cell ( $\times 100\,000$ ). Gold particles of 10 nm indicate the presence of the enzymes on the membranes of the tubulovesicular system (small arrows), and on the apical membrane (large arrows). (**c, d**) Interdigitation of basolateral membranes of the parietal cells ( $\times 70\,000$ ). BB-CK-specific labelling (**c**) but not  $H^+/K^+$ -ATPase labelling (**d**) is seen. (**e, f**) Simultaneous labelling with antibodies specific for CK (5 nm gold) and  $H^+/K^+$ -ATPase (10 nm gold) showing co-localization of these enzymes on the apical membranes (**e**) and on the membranes of the tubulovesicular system (**f**) of the parietal cells ( $\times 100\,000$ ).

samples (Figure 2), including the most highly purified preparation, indicating that this enzyme can indeed bind specifically to the membranes of the tubulovesicular system. CK activity measurements confirmed that a small but significant portion of the enzyme was associated with the most highly purified membranous fraction (Figure 2). No MM-CK and MB-CK isoforms were observed in these fractions (results not shown).

To test whether tubulovesicular-system-bound BB-CK was able to provide  $H^+/K^+$ -ATPase with high-energy phosphoryl groups, a system was used in which  $K^+$ -loaded vesicles were suspended in a buffer containing the fluorescent dye AO. As AO is an uncharged molecule that can cross the vesicle membrane, equilibration of intravesicular and extravesicular AO concen-

trations will occur. Proton uptake resulting from vesicle-bound  $H^+/K^+$ -ATPase activity will result in intravesicular acidification and in the consequent formation of  $AO^+$  ions that cannot cross the vesicle membrane. Extravesicular AO molecules will then enter the vesicles to re-establish the AO equilibrium.  $H^+/K^+$ -ATPase activity can be monitored by the decrease in AO fluorescence, because the increasing intravesicular concentrations of AO cause quenching of the fluorescence signal [29].

In the first set of experiments, 1 mM PCr was used as the sole energy source and the reaction was started by the addition of 100  $\mu$ M ADP. As shown in Figure 3(a) (left panel),  $H^+/K^+$ -ATPase activity was clearly detectable, thus indicating that ATP generated by BB-CK supports  $H^+/K^+$ -ATPase activity. Addition



**Figure 2** Immunodetection on Western blots and determination of enzyme activity for BB-CK and  $H^+/K^+$ -ATPase in hog gastric vesicles of increasing purity

A sample of 5  $\mu$ g of protein from each successive fraction from the vesicle purification procedure [26] was electrophoresed and blotted on to Immobilon-P membranes. Blots were incubated with specific antibodies for  $H^+/K^+$ -ATPase (a) and CK (b), and stained as described in the Materials and methods section. Controls are baculovirus-produced BB-CK (0.5  $\mu$ g) [22] and  $H^+/K^+$ -ATPase (10  $\mu$ g) [36]. CK activities (units/mg of protein) of all samples are indicated on top of each lane. Positions of the markers (in kDa) are indicated on the right. Lane 1, 20 000 g supernatant; lane 2, 100 000 g supernatant; lane 3, 100 000 g pellet (microsomal fraction); lane 4, 37% sucrose/Ficoll interface (fraction A, containing a mixture of various structures, open membranes, endoplasmic reticulum and vesicles); lane 5, 0.25 M sucrose/Ficoll interface (fraction B, containing mostly unilamellar vesicles); lane 6, osmotic shock preparation of fraction B; lane 7, recombinant BB-CK; lane 8, recombinant  $H^+/K^+$ -ATPase. Note the presence of large amounts of BB-CK in lane 2, presumably representing the unbound cytosolic fraction of the enzyme.

to the vesicles of ADP alone showed no effect (results not shown), proving that the ATPase activity was not a consequence of ATP generated by traces of adenylate kinase (myokinase). A second set of experiments was performed with 1 mM phosphoenolpyruvate (PEP) as energy donor and pyruvate kinase (PK) as ATP-generating enzyme. The reaction was once more started by the addition of 100  $\mu$ M ADP, and again distinct  $H^+/K^+$ -ATPase activity was observed. The PK concentration was chosen such that  $H^+/K^+$ -ATPase activity was the same for both the CK- and the PK-driven systems (Figures 3a and 3b, left panels). In order to compare the efficacy of the two energy supply systems under these conditions, the rate of ATP synthesis was determined in the presence of SCH 28080, an agent that specifically blocks  $H^+/K^+$ -ATPase activity [30], using a hexokinase/G6PD-based ATP trap. The results show that the relative rate of ATP synthesis in the CK system is only 10% of that of the PK system, under conditions where the  $H^+/K^+$ -ATPase activities in the absence of SCH 28080 are the same for both systems (Figures 3a and 3b, black bars).

In order to verify these results, the PK concentration was lowered to equalize the rates of ATP synthesis of the PK- and CK-driven systems (Figures 3a and 3c, black bars). This resulted in a clear decrease in  $H^+/K^+$ -ATPase activity (Figure 3c, left panel). Addition of excess ATP (250  $\mu$ M) after 4 min resulted in a clear and immediate decrease in the PK system fluorescence, indicating that under these conditions  $H^+/K^+$ -ATPase was not able to remove all  $K^+$  from the vesicles within the experimental time span. In contrast, the addition of excess ATP after 4 min had no effect on the ATPase activity in the CK system (Figure 3a, left panel), indicating that sufficient ATP was generated for  $H^+/K^+$ -ATPase to clear  $K^+$  from the vesicles within this time.

The rate of ATP synthesis was also determined without the  $H^+/K^+$ -ATPase blocking agent SCH 28080, again using the hexokinase/G6PD-based ATP trap. As  $H^+/K^+$ -ATPase will now

use part of the ATP formed, an absolute decrease in the rate of ATP synthesis was expected which would be the same for both systems because of their equal  $H^+/K^+$ -ATPase activities. However, Figures 3(a) and 3(b) (open bars) indicate that the absolute decrease in the rate of ATP synthesis was much higher for the PK system than for the CK system. These results strongly suggest that the flow of high-energy phosphoryl catalysed by the membrane-bound fraction of BB-CK is driven by  $H^+/K^+$ -ATPase activity.

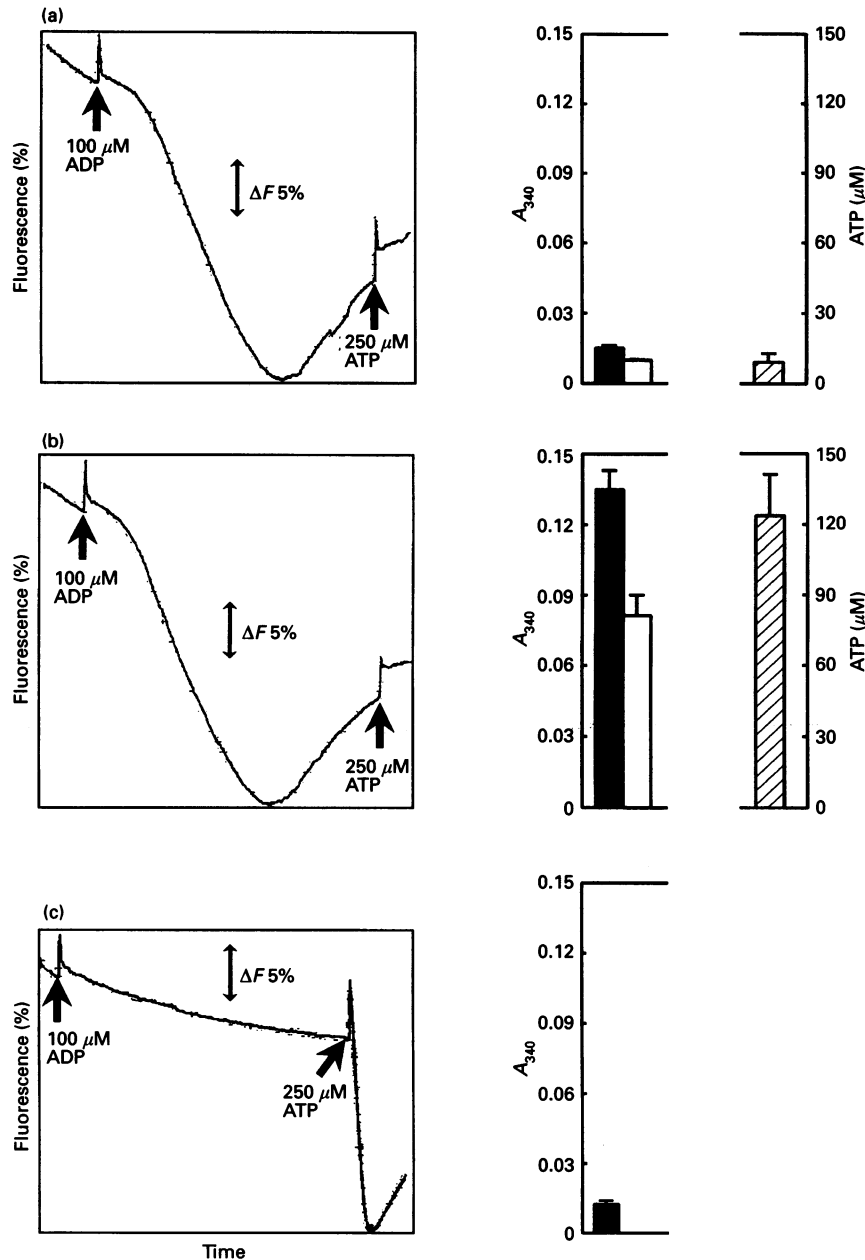
Finally, a luciferin/luciferase ATP-measuring system was used to determine the actual concentrations of free ATP in the PK and CK assay systems. ATP concentrations were measured at several time points during a 2.5 min reaction period, but were found to remain constant after 15 s (when the first sample was taken) in both systems. In Figures 3(a) and 3(b) the average concentrations during this interval are shown (hatched bars). Although the uptake profiles (Figures 3a and 3b, left panels) were very similar, the actual concentration of free ATP present in the CK system was only 10% of that of the PK system (Figure 3, hatched bars). In the PK system virtually all ADP was converted and maintained as ATP during the entire course of the incubation. As we know from the hexokinase trapping experiments that the rate of PK-driven ATP synthesis is linear during this period, we conclude that the capacity of the PK system exceeds the ATP-hydrolysing activity in the vesicle preparation. When the concentration of free ATP in the PK system was decreased to the levels observed in the CK system by decreasing the PK concentration,  $H^+/K^+$ -ATPase activity almost ceased (results not shown). These results thus confirm that BB-CK was much more effective in providing the  $H^+/K^+$ -ATPase with ATP than was PK, suggesting a functional coupling between BB-CK and  $H^+/K^+$ -ATPase on the vesicles of the parietal cells.

## DISCUSSION

BB-CK is expressed at very high levels in gastric parietal cells of humans, mice and rabbits [20,21]. We present here new data concerning the intracellular localization and the biochemical role of BB-CK in this highly specialized cell type. IEM studies using CK- and  $H^+/K^+$ -ATPase-specific antibodies clearly demonstrated the co-localization of BB-CK with gastric  $H^+/K^+$ -ATPase on the apical membrane and the membranes of the tubulovesicular system (Figure 1). BB-CK was also observed on the basolateral membranes, where  $Na^+/K^+$ -ATPase, which plays a crucial role in maintaining intracellular  $K^+$  concentrations, is located [15]. The close association of these two enzymes suggests that the CK/PCr system may play a role in  $Na^+/K^+$  homeostasis, analogous to the situation in the heart [10].

The co-localization of BB-CK and  $H^+/K^+$ -ATPase in rabbit parietal cells was confirmed when vesicles enriched in  $H^+/K^+$ -ATPase were isolated from hog parietal cells. Although most of the BB-CK was lost during the purification process, a significant fraction of both protein and enzymic activity remained, even after extensive rinsing followed by an osmotic shock. That CKs can interact with membranes has been demonstrated before, both with isolated vesicles of the sarcoplasmic reticulum [31] and with membrane models [32]. It is still uncertain whether specific modifications (i.e. phosphorylation) play a role in these associative distributions, although there is some evidence for the involvement of myristoylation [33].

Experiments were then performed to investigate the functional significance of the co-distribution of BB-CK and  $H^+/K^+$ -ATPase. Our results showed the vesicle-bound fraction of BB-CK to fuel gastric  $H^+/K^+$ -ATPase much more efficiently than an ATP-generating system based on PK. The method used for determining



**Figure 3** Comparisons between H<sup>+</sup>/K<sup>+</sup>-ATPase activity, the rate of ATP synthesis and free ATP concentration in the CK and PK ATP-generating systems

H<sup>+</sup>/K<sup>+</sup>-ATPase activity (left panels) was measured as the decrease in fluorescence due to the uptake of AD by the vesicles. The double-headed arrow indicates a 5% decrease in fluorescence. Reactions were started at  $t = 0$  (left arrow) by adding 100 μM ADP to the reaction mixture. At  $t = 4$  min, excess (250 μM) ATP was added (right arrow) to ensure that all K<sup>+</sup> ions were removed from the vesicles. The right-hand panels show histograms representing the rate of ATP synthesis as determined between 1 and 2.5 min with a hexokinase/G6PD-based ATP trap. Results are depicted in the presence (closed bars) and absence (open bars) of the H<sup>+</sup>/K<sup>+</sup>-ATPase blocking agent SCH 28080. Hatched bars represent the mean actual free ATP concentrations as measured between 15 s and 2.5 min with a luciferin/luciferase system. Experiments were performed in duplicate (a, b) or in triplicate (c). The S.E.M.s are indicated. (a) Results with the CK-driven ATP-generating system in the presence of 1 mM PEP. (b) Results with the PK-driven system in the presence of 1 mM PEP, with a PK concentration such that the H<sup>+</sup>/K<sup>+</sup>-ATPase activity equalled that of the CK system. (c) Same situation as (b), but with the PK concentration chosen such that the rate of ATP synthesis equals that of the CK system in the presence of SCH 28080.

the rate of ATP synthesis was based on a system driven by hexokinase, which can be regarded as a hexokinase-based ATP trap [34]. The fact that the rate of ATP synthesis measured in the CK system was very low, despite the clear H<sup>+</sup>/K<sup>+</sup>-ATPase activity, indicates that ATP generated by BB-CK is preferentially utilized by H<sup>+</sup>/K<sup>+</sup>-ATPase and is shielded from capture by hexokinase. In contrast, ATP generated by the exogenous PK

system is not preferentially used by H<sup>+</sup>/K<sup>+</sup>-ATPase and is thus accessible to hexokinase.

To exclude the possibility that the difference between the PK and CK systems was the result of specific interactions between PK or CK and hexokinase and/or G6PD, we measured free ATP concentrations using an independent luciferin/luciferase assay. Again, there was a clear difference between the two systems. In

the PK system the free ATP concentration was about 100  $\mu\text{M}$ , indicating that all the ADP that was added to start the reaction was converted into ATP within 15 s. However, in the CK system the signal hardly exceeded background levels, and ATP concentrations never exceeded 10  $\mu\text{M}$ . These results therefore support the results of the hexokinase assays, and again imply that there is functional coupling between BB-CK and  $\text{H}^+/\text{K}^+$ -ATPase.

In this regard, an interesting observation was made when the  $\text{H}^+/\text{K}^+$ -ATPase blocking agent SCH 28080 was added during the ATP synthesis measurements. A distinct increase in the rate of ATP synthesis was observed in the PK system, which can be explained by the fact that under these circumstances  $\text{H}^+/\text{K}^+$ -ATPase does not compete with hexokinase for free ATP. However, only a minor increase in the rate of ATP synthesis was observed in the CK system, although  $\text{H}^+/\text{K}^+$ -ATPase activities in the absence of SCH 28080 were the same for both systems. The finding that blocking  $\text{H}^+/\text{K}^+$ -ATPase results in at least a 10-fold greater increase in ATP availability in the PK system compared with the CK system strongly suggests that the BB-CK-catalysed flow of high-energy phosphoryl is kinetically linked to  $\text{H}^+/\text{K}^+$ -ATPase activity. This effect may be explained by the two enzymes forming a complex in which their activity is regulated in concert. However,  $\text{H}^+/\text{K}^+$ -ATPase can be activated *in vitro* by the sole addition of ATP, which may argue against this. Nevertheless, it should be noted that this is a model system that need not reflect exactly the situation *in vivo*. As BB-CK is not an integral membrane protein, a significant proportion of this enzyme may be lost during the purification process, leaving a fraction of  $\text{H}^+/\text{K}^+$ -ATPase not associated with BB-CK that can be activated by ATP. Alternatively, coupling of the two enzymes may be indirect, through equilibrium shifting of the  $\text{MgADP}^- + \text{PCr}^{2-} + \text{H}^+ \rightleftharpoons \text{MgATP}^{2-} + \text{creatine}$  reaction within a local unstirred (or Nernst) layer [35] at the protein-membrane interphase or any other putative microcompartment between the two enzymes. In this model the local accumulation of ATP, or more likely the increase in the ATP/ADP ratio, will impair the BB-CK conversion reaction. Whatever the exact nature of the phenomenon, our results clearly demonstrate the existence of a functional coupling, either direct or indirect, between BB-CK and  $\text{H}^+/\text{K}^+$ -ATPase.

Functional coupling between CKs and ATPases, i.e. preferential use of CK-generated ATP by the ATPases and, vice versa, preferential use of ATPase-generated ADP by the CKs, may serve to maintain ATP/ADP ratios in the close vicinity of ATPases that are favourable for their optimal function. Functional co-operation between CK enzymes and two members of the class of P-type ATPases, the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase [12,13] and  $\text{Na}^+/\text{K}^+$ -ATPase [10,11], has been described previously. Our findings on gastric  $\text{H}^+/\text{K}^+$ -ATPase, another P-type ATPase, further support the conclusion that functional association with members of the CK family is a common characteristic of this class of ATPases. However, it should be stressed that not all BB-CK is tightly associated with recognizable cellular structures, and the presence of high levels of unbound CKs in gastric parietal cells suggests an additional role for the CK system in these cells. This was unexpected, as there is no obvious need to compensate for or buffer very rapid changes in ATP demand, or to transport ATP across large cellular distances, in parietal cells. First, these cells contain more mitochondria than any other cell type, and consequently have a high ATP-generating capacity through oxidative phosphorylation. Secondly, although the parietal cells are polarized, the mitochondria are dispersed throughout the cytosol, with the majority being clustered close to the plasma membrane, i.e. close to the sites of high ATP turnover. This would suggest that the cell is already

optimally adapted to support energy requirements and a direct role for the cytosolic BB-CK fraction is therefore not immediately evident. However, Ekblad [19] has shown that the apparent equilibrium constant of the CK reaction increases during gastric acid secretion, thereby directly relating BB-CK activity with the main physiological function of parietal cells. Soluble BB-CK activity may thus serve to dampen extreme fluctuations in intracellular pH and ATP/ADP ratios that occur during the energy-demanding proton transport process. The relevance and possible dynamics of the partitioning of BB-CK between soluble and membrane fractions in response to fluctuations in metabolic demands in these cells needs further clarification.

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